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Abstract: BACKGROUND Strategies aiming at minimization or elimination of systemic immunosuppression are key immediate goals for clinical expansion of vascularized composite allotransplantation (VCA). We compared the in vitro and in vivo immunomodulatory efficacy of adipose-derived mesenchymal stem cells (AD-MSCs) and bone marrow (BM)-derived MSCs in a rat VCA model. METHODS Both cell types were tested in vitro for suppressor function using mixed lymphocyte reactivity assays. AD-MSCs or BM-MSCs were administered intravenously (1×10 or 5×10 cells/animal) to Lewis rat recipients of mismatched Brown Norway hindlimb transplants. Short course tacrolimus (FK-506) monotherapy was withdrawn at postoperative day 21. In vivo regulatory T-cell induction, peripheral blood chimerism, and microchimerism in lymphatic organs were analyzed. RESULTS AD-MSCs and BM-MSCs exhibited strong dose-dependent suppressor function in vitro, which was significantly more pronounced for AD cells. In vivo, all animals revealed peripheral multi-lineage chimerism at four weeks ($P < 0.01$) independent of cell type and dosage. Regulatory T-cell levels were increased with both cell types, the most in AD-MSC groups. These immunomodulatory effects were only transient. MSC treatment resulted in long-term (>120 day) allograft survival in 47% of the animals, which correlated with durable microchimerism in BM and spleen. CONCLUSIONS AD-MSCs and BM-MSCs exert immunomodulatory effects that prolong survival of immunogenic skin-bearing VCA grafts with short course (21 day) tacrolimus induction therapy. The in vivo findings in terms of allograft survival did not reflect superior immunomodulatory characteristics of AD-MSCs found in vitro.

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Adipose- and Bone Marrow–Derived Mesenchymal Stem Cells Prolong Graft Survival in Vascularized Composite Allotransplantation

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Background. Strategies aiming at minimization or elimination of systemic immunosuppression are key immediate goals for clinical expansion of vascularized composite allotransplantation (VCA). We compared the in vitro and in vivo immunomodulatory efficacy of adipose-derived mesenchymal stem cells (AD-MSCs) and bone marrow (BM)–derived MSCs in a rat VCA model. **Methods.** Both cell types were tested in vitro for suppressor function using mixed lymphocyte reactivity assays. AD-MSCs or BM-MSCs were administered intravenously (1×10^6 or 5×10^6 cells/animal) to Lewis rat recipients of mismatched Brown Norway hindlimb transplants. Short course tacrolimus (FK-506) monotherapy was withdrawn at postoperative day 21. In vivo regulatory T-cell induction, peripheral blood chimerism, and microchimerism in lymphatic organs were analyzed. **Results.** AD-MSCs and BM-MSCs exhibited strong dose-dependent suppressor function in vitro, which was significantly more pronounced for AD cells. In vivo, all animals revealed peripheral multi-lineage chimerism at four weeks ($P < 0.01$) independent of cell type and dosage. Regulatory T-cell levels were increased with both cell types, the most in AD-MSC groups. These immunomodulatory effects were only transient. MSC treatment resulted in long-term (>120 day) allograft survival in 47% of the animals, which correlated with durable microchimerism in BM and spleen. **Conclusions.** AD-MSCs and BM-MSCs exert immunomodulatory effects that prolong survival of immunogenic skin-bearing VCA grafts with short course (21 day) tacrolimus induction therapy. The in vivo findings in terms of allograft survival did not reflect superior immunomodulatory characteristics of AD-MSCs found in vitro.

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Greater than 30 face and 100 upper extremity human vascularized composite allotransplantation (VCA) procedures have been performed around the world in the past 15 years.[1-3] Although recently defined as solid organ transplantation (SOT) by UNOS, VCA are inherently different in that they are life-enhancing rather than life-saving grafts, with clinical success determined not only by graft survival but also by neurofunctional outcomes.[4] The chronic risks of immunosuppression must be minimized or eliminated to achieve equipoise in VCA and facilitate clinical expansion. Novel cellular therapies that combine the benefits of immunoregulation with neuroregeneration could optimize immune, functional, and patient quality of life outcomes of these reconstructive modalities.[5]

Bone marrow (BM)–derived or adipose-derived (AD) mesenchymal stem cells (MSCs) have emerged as promising cell therapies for immunomodulation.[6] Compared to BM, adipose tissue is a richer source of MSCs with up to 10-fold higher cell yields.[7] The AD-MSCs possess superior immunomodulatory and suppressor potential in vitro compared to

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BM-MSCs.[8,9] Mesenchymal stem cells have been successfully used to facilitate hematopoietic stem cell engraftment,[10,11] in clinical SOT,[12,13] and treatment of graft-versus-host disease.[14,15] Unlike most SOT, VCA poses a stringent challenge because of the differential antigenicity of tissues and the high immunogenicity of skin. However, early studies in preclinical models demonstrate that MSCs are indeed immunomodulatory in VCA, driving the momentum for active research efforts in this area.[16]

We evaluated the dose-dependent suppressor effect in vitro and immunomodulatory efficacy in vivo of BM-MSCs and AD-MSCs in a rodent VCA model.

MATERIALS AND METHODS

All experiments were approved and performed in compliance with University of Pittsburgh Institutional Animal Care and Use Committee guidelines.

MSC Isolation

Inguinal adipose tissue was obtained from 6- to 8-week-old Brown Norway (BN) rats and enzymatically digested with collagenase type II (Worthington Biochemical Corp, Lakewood, NJ) and bovine serum albumin (Millipore, Billerica, MA) in Hanks balanced saline solution (Cellgro Mediatech Inc. Manassas, VA) for 60 minutes at 37 °C. After centrifugation, the cellular pellet (stromal vascular fraction) was erythrocyte-depleted by lysis buffer, filtered, and transferred complete medium (Dulbecco's modified eagle's medium/F-12, 10% fetal bovine serum, 1% penicillin/streptomycin, 1.25 mg/L amphotericin-B [all Gibco, Grand Island, NY], 0.1 μM dexamethasone [Sigma-Aldrich, St. Louis, MO]). The AD-MSCs were allowed to attach for 6 hours, washed with phosphate-buffered saline and expanded until passage 3.

The BM-MSCs were obtained from 6- to 8-week-old BN rats by flushing long bones with Roswell Park Memorial Institute medium 1640 (Lonza, Walkerville, MD). The cellular pellet was erythrocyte-depleted, centrifuged, resuspended in complete medium (Roswell Park Memorial Institute medium 1640, 10% fetal bovine serum, 2.5 μM HEPES [Sigma-Aldrich], 1% penicillin-streptomycin, 1.25 mg/L amphotericin B, 1% L-glutamine, 1% sodium pyruvate, and 1% 2-mercaptoethanol [all Gibco]) and expanded in vitro until passage 3. For injections, MSCs were lifted, counted and resuspended (1×10^6 and 5×10^6 cells) in 1 mL of phosphate-buffered saline for injection.

Characterization of MSC

Expanded cells were stained for CD29, CD73, CD90, CD45, and RT1b (MHC I) and analyzed using a fluorescence-activated cell sorter (Aria, Becton Dickinson, Franklin Lakes, NJ). Data were analyzed using FlowJo software (TreeStar Inc., Ashland, OR).

Mixed Lymphocyte Reaction Assays

Responder T cells were isolated from blood, spleens, or lymph nodes of naive rats (Lewis [LEW]), and stimulator cells were isolated from spleens of donor rats (BN). Responder and stimulator cell suspensions were erythrocyte-depleted by lysis buffer. Stimulators were irradiated at 3000 rads.

Responder peripheral blood mononuclear cells (PBMCs) were isolated from whole blood of naive LEW rats using gradient density centrifugation method, whereas BN MSCs were isolated as described above. T cells were isolated by T-cell enrichment columns (R&D Systems, Minneapolis, MN), and cell purity was confirmed to be greater than 90% by flow cytometry for $CD3^+$ cells.

In the primary suppressor assay, T-cells (2×10^5 cells/well) were cocultured for 7 days with either BN AD-MSCs or BM–MSCs (T-cell to AD-MSC/BM-MSC ratio of 1:1, 4:1, 8:1, and 16:1) in triplicates in round-bottom 96-well plates, in the presence of irradiated naïve BN splenocytes (5×10^4 cells/well).

In the secondary suppressor assay, LEW PBMCs (2×10^5 cells/well) were cocultured for 72 hours with BN AD-MSCs or BM-MSCs (PBMCs to AD-MSC/BM-MSC ratio of 1:1, 2:1, 4:1 and 8:1) while stimulated with anti-CD3 and anti-CD28 monoclonal antibodies (each 2 μg/mL).

Finally, to assess T-cell proliferation, cells in both assays were pulsed with [^{3}H]thymidine (1 mCi/well) for the final 8 hours and [^{3}H]thymidine incorporation was measured as counts per minute (CPM) in a liquid scintillation counter (Perkin Elmer). Six experiments were performed for both mixed lymphocyte reaction (MLR) assays.

Animals

Six- to 8-week-old male LEW ($RT1^1$, recipient) and BN ($RT1^n$, donor) rats weighing 250 to 300 g were purchased from Harlan (Indianapolis, IN) and maintained in a specific pathogen-free environment at the University of Pittsburgh.

Surgical Technique and Procedural Detail

Lewis rats received orthotopic hindlimb transplants from BN donor rats. Isoflurane was used for inhalation anesthesia. Bilateral shaved hind limbs of BN donors were dissected through a circumferential skin incision, after ligation of epigastric vessels, dissection of femoral vessels, and transection at the level of the inguinal ligament. The 2 limbs were amputated at midfemoral level and served as donor limbs for 2 LEW recipients. Osteosynthesis was performed using an intramedullary 18-gauge needle. Sciatic nerves were coapted using 9-0 nylon sutures (Microsurgery Instruments, Inc., Bellaire, TX) and the muscles sutured with interrupted 4-0 Vicryl stitches (Ethicon Inc., Somerville, NJ). Microsurgical anastomosis of the femoral artery was performed with interrupted 11-0 nylon stitches, whereas a polyamide tube (RiverTech Medical, Chattanooga, TN) was used as cuff for the femoral vein, as reported earlier.[17]

Experimental Protocol

Recipient animals were assigned to 5 groups: controls (n = 5), 1×10^6 BM-MSCs (n = 6), 5×10^6 BM-MSCs (n = 7), 1×10^6 AD-MSCs (n = 8), and 5×10^6 AD-MSCs (n = 9). All recipients were treated with rabbit antirat lymphocyte serum (CEDARLANE, Burlington, NC) 4 days before and 1 day after surgery. Daily immunosuppression with tacrolimus (FK-506; 0.5 mg/kg) was administered intraperitoneally from day 0 to postoperative day (POD) 21. The treatment groups received a single-shot MSC injection intravenously on POD 1, injected slowly through the penile vein using a 30-gauge needle. Animals were observed daily for signs of rejection, assessed according to clinical VCA rejection grading which is based on graft gross appearance.[18] When rejection grade III was reached (endpoint), animals were sacrificed for tissue sampling. In 2 nonrejecting animals, BN donor skin grafts were transplanted to the neck to confirm donor-specific tolerance after 120 days.

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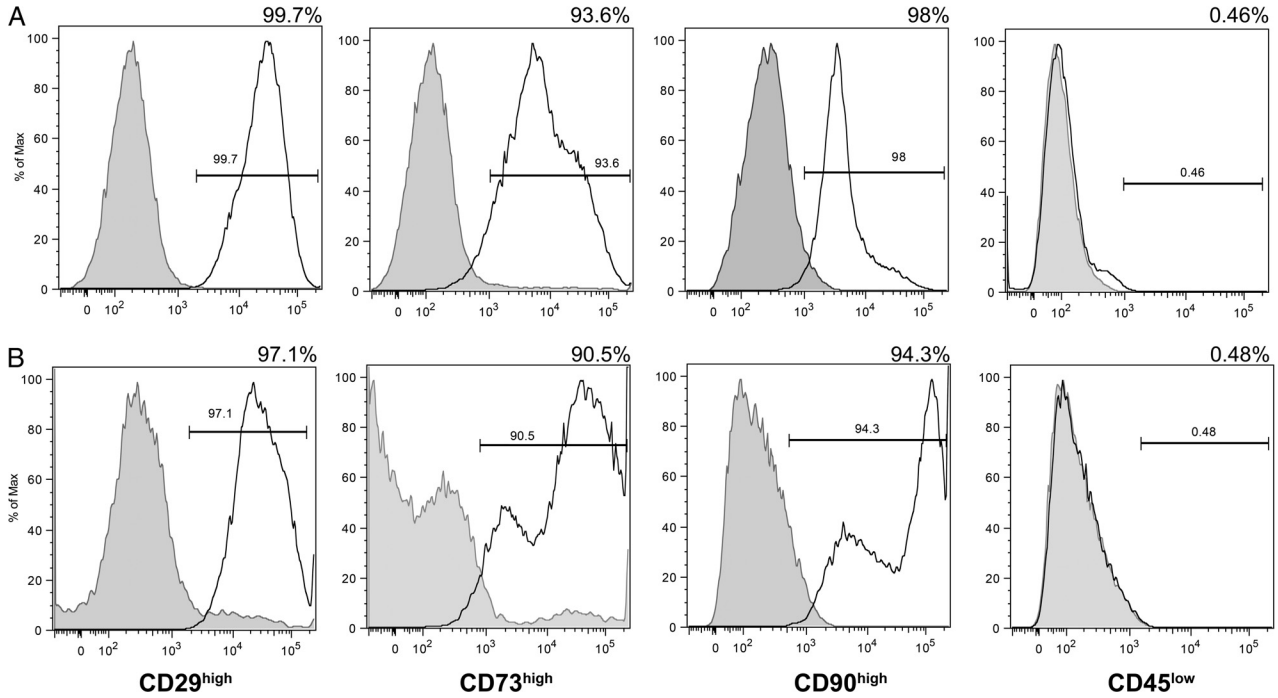

FIGURE 1. MSCs were characterized before cell-based immunosuppressive therapy according to surface marker expression. BM-MSCs (A) and AD-MSCs (B) showed similar expression profiles ($CD29^{hi}CD73^{hi}CD90^{hi}CD45^{low}$).

Analysis of Regulatory T Cell and Chimerism

Chimerism and regulatory T-cell (T_{reg}) levels were assessed in peripheral blood on PODs 28 and 42 in MSC-treated animals (n = 14 in AD-MSC groups and n = 9 in BM-MSC groups). As controls (n = 5) rejected early, blood tests were only possible at 4 weeks. Peripheral blood, lymph nodes, spleens, thymus, and BM of recipient LEW rats were collected at euthanasia in long-term survivors (>120 days; BM-MSC groups [n = 3]; AD-MSC groups [n = 6]) to analyze microchimerism.

For T_{reg} analysis, red blood cells were erythrocyte-depleted and remaining cells stained with various cell surface markers (fluorochrome-conjugated mouse anti-rat CD3, CD4, CD8, CD25, CD11b/c, CD45RA antibodies) or intracellular markers (fluorochrome-conjugated mouse antirat Foxp3 antibody).

Peripheral blood and lymphatic tissue chimerism was evaluated with mouse antirat class I RT1Ac antibodies (AbD Serotec) were used for donor cell labeling ($RT1^n$, BN rats) and mouse antirat CD45Ra, CD11b/c, CD4, and CD8 antibodies (eBioscience) were used as markers of cell lineages.

All cells were then analyzed using flow cytometry (LSRII) and data analyzed using FlowJo software (TreeStar Inc., Ashland, OR).

Histopathology

Upon euthanasia, component tissues from recipient graft explants were formalin-fixed and paraffin-embedded. Four-µm sections were stained with hematoxylin-eosin, graded by blinded pathologists based on the Banff classification,[19] digitally photographed at 10× magnification and correlated with clinical photographs of limbs at varying time points in the rejection course.

Statistical Analysis

InStat and Prism software (GraphPad Software, La Jolla, CA) were used for statistical analysis. The data are presented as means ± SD unless otherwise indicated. Differences between groups were assessed by unpaired analysis of variance. Graft survival was compared between the different groups using log-rank (Mantel-Cox) test and represented as Kaplan-Meier curve. A value of P less than 0.05 was considered statistically significant.

RESULTS

Characterization of AD-MSC and BM-MSC

The MSCs from BM and adipose tissue were characterized using flow cytometry (Figure 1). Both cell lines were negative for the hematopoietic marker CD45 (0.46% for BM-MSCs [Figure 1A] and 0.48% for AD-MSCs

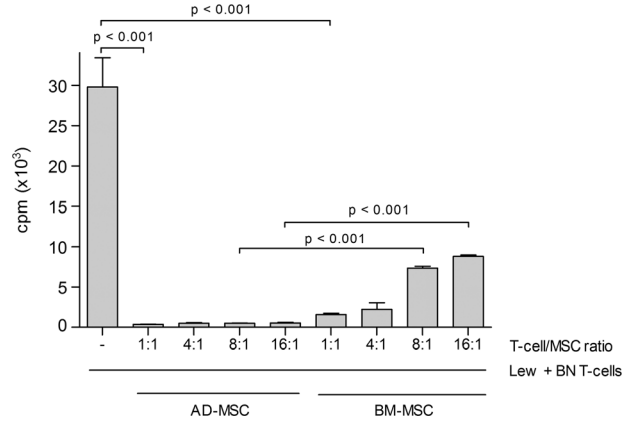

FIGURE 2. MLR with LEW T cells (recipient) stimulated with BN irradiated splenocytes (donor). Although LEW T cells showed high alloresponsiveness after the addition of donor cells, this was widely suppressed in the presence of AD-MSCs. BM-MSCs showed dose-dependent immunosuppression, which was less extensive than with AD-MSCs. Represented in CPM.

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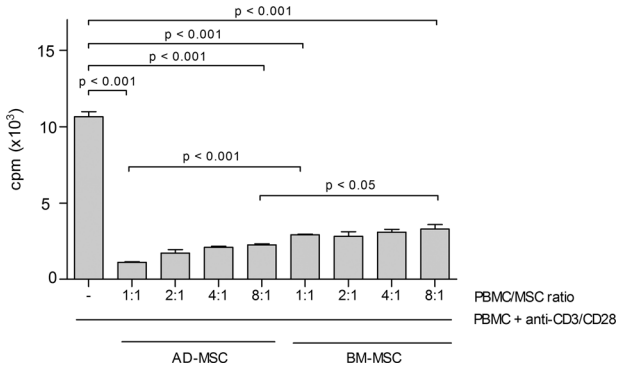


FIGURE 3. MLR with PBMC under anti-CD3/CD28 stimulation. Although stimulated PBMCs from naive animals showed an extensive response (control [N]), MSCs were able to suppress this effect. The immunosuppressive effect was dose-dependent and more pronounced in the AD-MSC than BM-MSC groups. Represented in CPM.

[Figure 1B]). Both cell types expressed high levels of CD29 (99.7% vs 97.1%), CD90 (98.0% vs 94.3%), and CD73 (93.6% vs 90.5%).

Analysis of Suppressor Activity In Vitro by MLR Assay

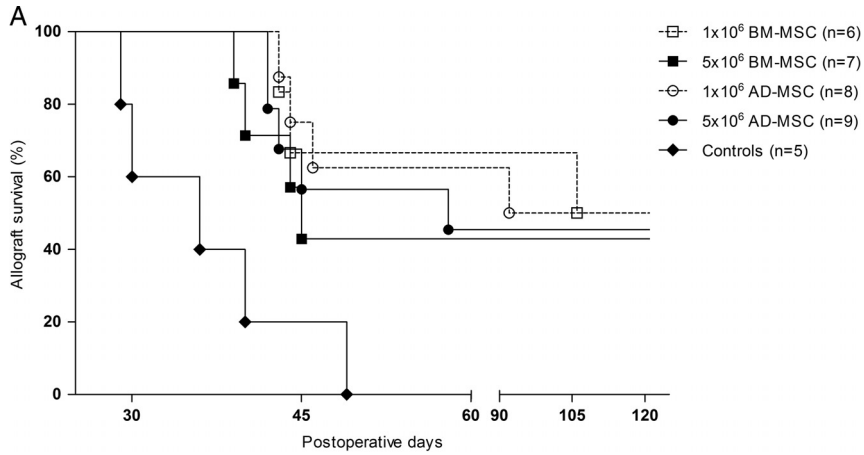
High proliferation was observed when recipient (LEW) T cells were stimulated with donor (BN) splenocytes, ($29.8 \pm 3.6 \times 10^3$ CPM [Figure 2]). The LEW T cells and BN splenocytes were cultured alone as negative controls, and no proliferation was observed (data not shown). Adding AD-MSCs to stimulated LEW T cells inhibited proliferation ($0.35 \pm 0.04 \times 10^3$ CPM at 1:1, $0.54 \pm 0.07 \times 10^3$ CPM at 16:1, all $P < 0.001$ vs. LEW + BN). BM-MSCs showed dose-dependent suppression: if cocultured 1:1, LEW T-cell proliferation was $1.6 \pm 0.2 \times 10^3$ CPM, increasing up to $8.8 \pm 0.2 \times 10^3$ CPM at a 16:1 ratio (all $P < 0.001$ vs. LEW + BN). The difference between AD-MSC– and BM-MSC–related suppression was significant at both 8:1 and 16:1 ratios ($P < 0.001$).

In a secondary assay, PBMCs from naive animals were highly responsive to stimulation with antibodies against CD3/CD28 ($10.7 \pm 0.3 \times 10^3$ CPM [Figure 3]). Unstimulated PBMCs were taken as negative control and did not proliferate (data not shown). Stimulated PBMCs (sPBMCs) were significantly suppressed in a dose-dependent manner after addition of AD-MSCs or BM-MSCs. At a 1:1 ratio (sPBMCs: MSCs), values of $1.1 \pm 0.03 \times 10^3$ CPM were obtained for AD-MSCs ($P < 0.001$ vs sPBMCs), whereas suppression was less extensive for BM-MSCs at a 1:1 ratio ($2.9 \pm 0.04 \times 10^3$ CPM, $P < 0.001$ vs both AD-MSCs and sPBMCs). At an 8:1 ratio, results were $2.3 \pm 0.08 \times 10^3$ and $3.3 \pm 0.03 \times 10^3$ CPM for AD-MSCs and BM-MSCs, respectively ($P < 0.001$ vs sPBMCs; $P < 0.05$ AD-MSCs vs BM-MSCs).

Allograft Survival and Tolerance

Controls consistently rejected their grafts before POD 50 ($P < 0.05$ vs BM-MSC/AD-MSC; Figure 4A). Variation in cell type or dosage did not correlate with significant differences in rejection or graft survival. Eight limbs of 17 in the AD-MSC

A



B

Groups	n=	Rejection (n=)		Long-term Survival (n=)		% Survival
		< POD 60	> POD 60 < POD 120	> POD 120		
BM-MSC 1x10⁶	6	2	1	3	BM-MSC	47% (6/13)
BM-MSC 5x10⁶	7	3	1	3		
AD-MSC 1x10⁶	8	3	1	4	AD-MSC	47% (8/17)
AD-MSC 5x10⁶	9	5	0	4		
Controls	5	5	-	-		0% (0/5)

FIGURE 4. Overall allograft survival curve (A) and data (B) for AD-MSC- and BM-MSC–treated animals (n = 30). All animals received ALS conditioning and immunosuppression with FK-506 from POD 0 until POD 21. Control animals (n = 5) completely rejected (grade III) the graft within 50 days, whereas cell-treated groups showed prolonged survival. Almost half (47%) of the MSC-treated animals exhibited long-term survival of the allograft (>120 days). ALS indicates antilymphocyte serum.

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group and 6 limbs out of 13 in the BM-MSC group were rejected by POD 60, followed by an additional allograft rejection in both AD-MSC and BM-MSC groups before POD 120. Overall, 47% of the transplanted limbs survived long-term beyond 120 days (8/17 for AD-MSC and 6/13 for BM-MSC; Figure 4B). Rejection grade in these animals was Banff 0-1. In 2 long-term survivors (n = 1 for each AD-MSC and BM-MSC group), secondary BN rat skin grafts were rejected within 20 days.

Chimerism

Peripheral blood multilineage chimerism was detected after 4 weeks in all animals treated with AD-MSCs and BM-MSCs (Figure 5A-C). Although controls showed no chimerism (0.65 ± 0.72%), levels for AD-MSC/BM-MSC–treated animals ranged between 5.06 ± 2.41 and 6.71 ± 1.52% ($P < 0.01$ vs control). Results did not differ between the groups receiving MSCs (dosage or cell type). Peripheral blood chimerism levels decreased with time and were 0.35 ± 0.28 and 0.98 ± 1.22% by 6 weeks, representing a significant drop ($P < 0.01$ vs at 4 weeks in all groups). Loss of peripheral chimerism was observed in early rejectors as well as long-term survivors. Blood samples, lymphatic organs, and BM were analyzed at endpoints for detection of microchimerism in long-term survivors (>120 days) (Figure 6). In the peripheral blood and thymuses of AD-MSC and BM-MSC groups, levels were below 1%. Inguinal lymph nodes of BM-MSC animals showed 2.79 ± 1.63% microchimerism on the transplanted side, whereas animals receiving AD-MSCs showed lower levels (1.04 ± 1.14%). In the spleen, AD-MSC animals revealed microchimerism with a percentage of 5.43 ± 2.09% compared to 3.00 ± 1.44% in BM-MSC animals. Microchimerism was detected in the tibial BM of the transplanted limb with 4.23 ± 0.72% in the BM-MSC group and 4.74 ± 1.87% in the AD-MSC group. Bone marrow of nontransplanted recipient tibias also exhibited microchimerism (3.17 ± 0.26% and 3.19 ± 0.54% for BM-MSC and AD-MSC group, respectively).

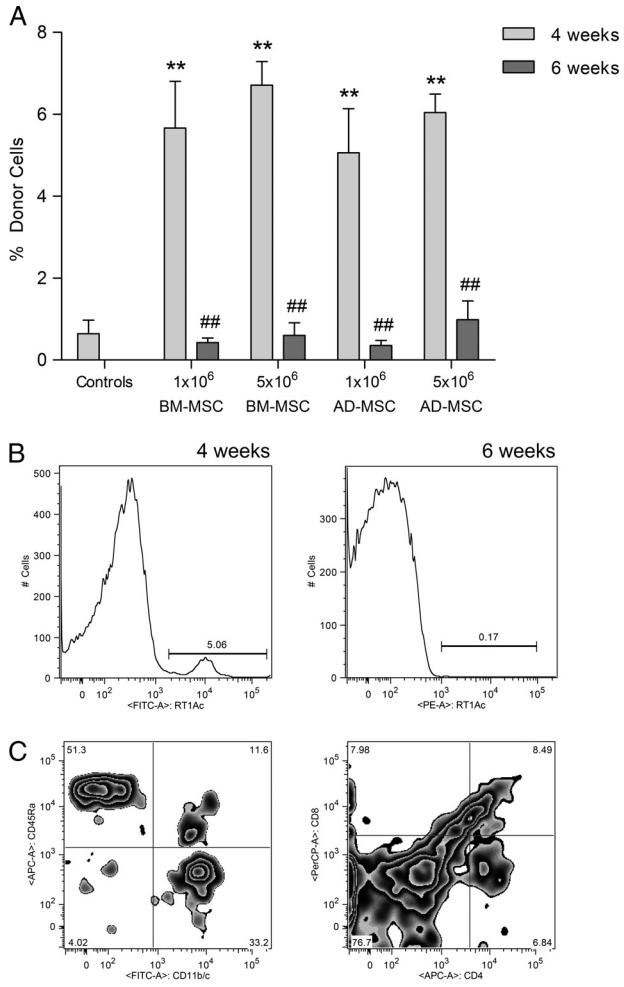

FIGURE 5. Levels of peripheral blood chimerism 4 and 6 weeks after hindlimb transplantation and MSC administration (A). All controls exhibited rejection at 6 weeks; therefore only data from week 4 are available. All MSC-treated groups showed significant levels of chimerism based on the percentage of MHC class I antigen positive population of all peripheral blood cells at 4 weeks. Yet this was only a transient event and was completely abolished after 6 weeks. Representative FACS image for peripheral blood chimerism based on class I RT1Ac antigen at 4 and 6 weeks (B; 1 × 10^6 AD-MSC group). (C) Further FACS analysis on RT1Ac$^+$ cells revealed multi-lineage chimerism based on CD11b/c/CD45R and CD4/CD8 antigens. **$P < 0.01$ vs. control; ##$P < 0.01$ versus 4 weeks. FACS indicates fluorescence-activated cell sorter.

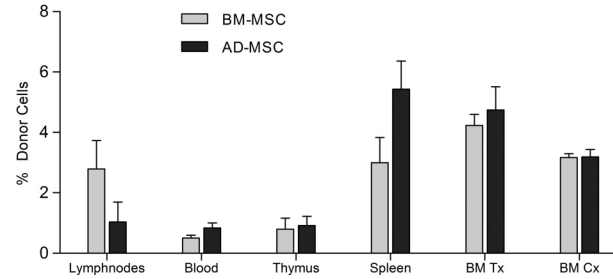

FIGURE 6. Peripheral microchimerism in immunologically relevant organs (inguinal lymph nodes, peripheral blood, thymus, spleen, and bone marrow in transplanted [BM Tx] and contralateral hindlimb [BM Cx]) based on class I RT1Ac antigen detected by FACS in animals with long-term allograft survival (>120 days). Although the levels were below 1% in the peripheral blood and thymus in these endpoint experiments, intermediate (1-3%) and inconsistent levels were found in inguinal lymph nodes on the allotransplant side. In spleen and bone marrow of the allograft (i.e., tibial bone) levels ranged as high as 3% to 5.5%. In the contralateral hindlimb, bone marrow levels were around 3%.

Regulatory T cells

The results for AD-MSCs and BM-MSCs were grouped for comparison because no significant differences were noted in outcomes for individual dosages. Four weeks after transplantation, there were 4.00 ± 1.04% T_{regs} in the control group (Figure 7A-B). Significantly higher levels (8.28 ± 3.60%) were only reached in the AD-MSC group ($P < 0.01$ vs. control). After 6 weeks, T_{reg} levels significantly dropped in the AD-MSC and BM-MSC groups ($P < 0.01$ vs 4 weeks) and remained lower at later time points as compared to 4 weeks, but higher than that in all animals at 6 weeks, including those that were prone to rejection.

Histopathology

Untreated controls (Figure 8E) showed histological signs of acute rejection in hematoxylin-eosin–stained skin samples and correlated with gross appearance found in vivo (clinical

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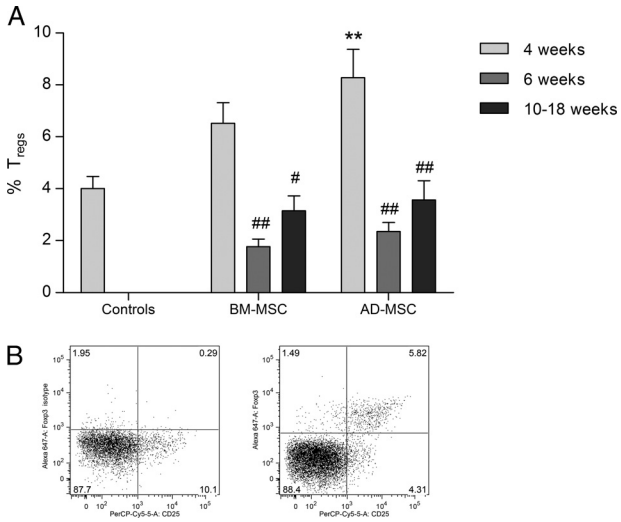

FIGURE 7. AD-MSCs and BM-MSCs induced T_{regs} ($CD4^+CD25^{hi}FoxP3^+$), which at week 4 was significant only for the AD-MSC group (A). High levels of T_{regs} were only transient and dropped significantly to lower levels after 6 weeks. In animals showing prolonged graft survival, we found slightly higher levels of T_{regs} compared to the 6-week time point (including the animals with rejection). $^{**}P < 0.01$ versus control; $\#P < 0.05$, $\#\#P < 0.01$ versus 4 weeks. (B) Representative FACS image of an animal with increased T_{regs} levels (right) and corresponding isotype control (left).

grade III). These included dense dermal, subdermal, and perivascular infiltrates with epidermal and adnexal involvement in terms of dyskeratosis, epithelial apoptosis as well as keratinolysis. Moreover, we found infiltration of adnexae with apoptosis and loss of architecture. Skin samples procured at end points from MSC-treated animals with long-term surviving allografts (Figure 8A-D) showed minimal or no signs of rejection in the histological assessment, regardless of cell dosage, confirming clinical findings. Histopathological grading per Banff criteria was limited to rare mild perivascular infiltrates in some samples, with no involvement of the overlying epidermal and adnexal structures.

DISCUSSION

This is the first direct comparison of the immunomodulatory effects of AD-MSCs and BM-MSCs in VCA. Our principal finding was that AD-MSCs and BM-MSCs, despite differences in in vitro suppressor activity, demonstrated similar immunologic efficacy and graft survival outcomes in vivo, after single administration. Around 50% of animals showed long-term allograft survival beyond 120 days after MSC treatment regardless of the source and dosage of cells. Our results confirm that graft acceptance rather than tolerance was achieved in treated recipients.

The MSCs from adipose tissue and BM revealed similar surface marker profiles, and were $CD29^{hi}CD73^{hi}CD90^{hi}CD45^{low}$, which is a generally accepted panel for mesenchymal stem cells.[20] In MLR assays, MSCs strongly suppressed T-cell proliferation in vitro. We were able to demonstrate in a head-to-head comparison that AD-MSCs possess superior suppressor potential than BM-MSCs. The BM-MSCs showed a dose-dependent suppression of alloantigen-induced T-cell proliferation, whereas AD-MSCs were able to almost completely abolish T-cell responses. The MLR assays with sPBMCs demonstrated similar suppressive potentials with both MSC types. These findings agree with recent reports in the literature,[21-23] but must be confirmed for human cells, where dose-dependent suppression has been reported.[24] The inherent advantages of AD-MSCs, such as abundant fat tissue availability, ease of retrieval, and smaller cell morphology compared to BM-MSCs, support their role in clinical indications of relevance to AD-MSC therapies.[25] Further studies in preclinical models are required to establish the role and impact of multiple variables related to cell dosing, timing, passage, and collateral effects of preparatory regimens.[26,27]

Long-term graft survival with survival greater than 120 days with no maintenance drug immunosuppression was achieved in 47% of the treated animals. Histological

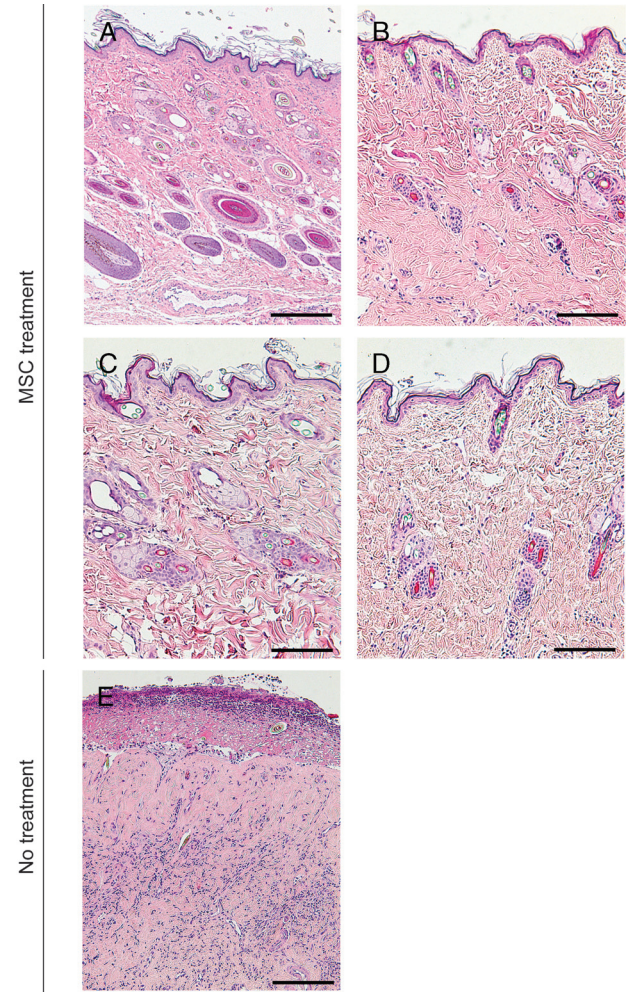

FIGURE 8. Histopathological findings in H&E-stained skin samples. BM-MSC- (1×10^6 group [A] and 5×10^6 group [C]) and AD-MSC (1×10^6 group [B] and 5×10^6 group [D]) treated long-term graft accepting animals showed no or minimal histological signs of rejection. The epidermal and dermal layers were well preserved with normal dermal papillae; the adnexae showed normal architecture and no infiltration. In few samples there was minimal perivascular infiltration. In MSC-untreated controls (E), we found keratinolysis and dyskeratosis as well as epithelial cell apoptosis, with loss of dermal papillae and an extensive infiltration throughout dermis and subdermis. Additionally, the adnexal structures showed inflammatory cell infiltration and apoptosis leading to architecture loss, which clinically correlated with hair loss as typical for grade II rejection or higher. 10× magnification. Bar, 250 μm. H&E indicates hematoxylin-eosin.

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grading of skin samples per the Banff classification confirmed rejection-free survival in all survivors.[19] Allograft survival may have been related to microchimerism in the BM and lymphatic organs as demonstrated by flow cytometry analysis, but this remains to be investigated further. We were not able to confirm tolerance in survivors, as donor-specific skin allografts were rejected in recipients with long-term hindlimb survival and no evidence of persistent thymic chimerism was observed, as reported to be a correlate or prerequisite for tolerance.[28] In clinical trials of BM transplantation, MSCs have been shown to facilitate hematopoietic stem cell engraftment without graft-versus-host disease.[29] In our study, donor cell engraftment per MHC class I characterization was observed not only in the transplanted limb but also in the contralateral unoperated recipient limb. Hematopoietic stem cells from the allograft BM may have engrafted into the recipient. It remains unknown if MSCs may have facilitated this process or engraft to lymphatic niches and modulate development of donor chimerism in that specific compartment.

Despite the fact that AD-MSCs and BM-MSCs revealed different immunosuppressive properties in vitro, the two cell types did not differ significantly in terms of long-term graft survival in vivo. Fundamental limitations of in vitro assays preclude them from being accurate reflectors of in vivo behavior of cells. For in vitro MLR, proliferation of T cells (either sorted or those in PBMC) in response to AD-MSCs and BM-MSCs was evaluated under controlled culture and incubation conditions. In contrast, the in vivo behavior of these cells is impacted by diverse and dynamic interactions between cellular and humoral systems as well as the kinetics of adaptive and innate immune phenomena. The in vivo environment is thus indeed a stringent test bed for confirmation of immunomodulatory efficacy. Our results indicate that neither variation of cell dosage nor cell type had a discernible advantage on long-term graft survival.

The MSC-based cell therapies have been studied in other rodent and swine VCA models.[30-32] Our experimental design in the current study was similar to that of Kuo et al,[32] although a different immunosuppressant (cyclosporine A) and 2 additional doses of AD-MSCs were administered, and a higher long-term graft survival was achieved. We used donor AD-MSCs in the current study. In contrast, Kuo et al, evaluated syngeneic AD-MSCs in VCA.[31] Their study suggests that repetitive AD-MSC injections do not offer additional survival advantage in VCA.[31] Regardless, a 66% VCA survival rate was achieved with donor-specific tolerance developed by syngeneic AD-MSCs, which is superior to that seen in our study with donor-derived cells. Immunologic outcomes and survival differences among these 3 studies may be related to immunogenicity of allogeneic MSCs. It is widely regarded that MSCs are immunoprivileged given their low expression of MHC class I and II evidenced by suppressor functions in vitro and in vivo.[33-35] However, there is also literature support for their capacity to induce alloantibodies, CD8-mediated cytotoxicity, or rejection in allogeneic settings.[36-38] Moreover, differentiation of MSCs in vitro during cell passage may also alter their immunogenic behavior.[39] Thus, cell longevity of allogeneic AD-MSCs may be different from syngeneic cells in vivo. If we hypothesize that donor-derived MSCs have a shorter in vivo lifespan, the benefits of multiple dosing of such cells become more evident as supported by the studies by Kuo et al[32] versus the results observed with single dose syngeneic AD-MSCs in VCA survival and tolerance.[31]

In our study, as in others, chimerism and T_{reg} upregulation were closely associated with prolongation of allograft survival.[28,40-42] A pattern of significant, yet transient upregulation of T_{regs} after MSC treatment was seen in our study, similar to that reported earlier by Kuo et al.[43] Cheng et al[31] achieved sustained circulating T_{reg} levels in long-term allograft surviving animals after the repetitive use of syngeneic AD-MSCs. T_{reg} induction has also been related to stable tolerance in the pertinent literature. The exact difference between syngeneic and allogeneic MSC in terms of immunomodulatory efficacy remains to be investigated.

Peripheral blood donor multilineage chimerism was found in MSC-treated recipients at an early stage (4 weeks), but was not maintained long term. However, investigation of lymphatic organs and BM revealed persistent donor microchimerism in animals with long-term acceptance of allografts. We interpret that sustained donor chimerism in various tissues could contribute to VCA acceptance, even without the benefit of sustained peripheral blood chimerism as found earlier by Kuo et al.[43] Chimerism may be an important requirement for maintenance of allograft tolerance, as shown by various groups in SOT.[44-46]

The MSCs have been shown to home to sites of injury and inflammation[47,48] as well as to transplanted skin,[49] whereas other groups described cell entrapment in filtering organs when injected systemically.[50,51] In the present study, indeed, we did not find any significant different outcome by increasing MSC dosage 5-fold (1×10^6 vs 5×10^6 cells), which may be related to the fact that MSCs exert many of their beneficial therapeutic effects through paracrine secretion overcoming the constraints of entrapment.[52,53] One of the primary limitations of the current study is lack of evidence for the collateral effect of immunosuppressive and lymphodepleting drugs on MSC function in vivo. We believe that the choice of conditioning and maintenance agents incorporated into transplant regimens as well as the timing, dosing, and duration of such treatments corresponding to or overlapping with cell-based therapies may significantly influence viability and function (proliferation, differentiation, homing, and engraftment) of cellular components. The effect of MSCs on functional motor and sensory outcomes was outside the scope of the study but should be addressed. These are all important research questions that must be systematically scrutinized in translational VCA studies. Optimization of conventional immunosuppressive regimens in VCA with MSC-based cell therapies warrants further investigation to improve safety, efficacy, and quality of life outcomes of these promising procedures.

CONCLUSIONS

Taken together, immunosuppression-free long-term VCA survival was achieved with both AD-MSCs and BM-MSCs. Peripheral blood chimerism and T_{reg} upregulation were only transient events. Our in vivo findings did not reflect the superior immunomodulatory characteristics of AD-MSCs in vitro.

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